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Development and Characterization of Polymeric Intravenous Nanoparticles encapsulating Low Molecular weight heparin.

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ABSTRACT
Objective: The objective of the current research work was to prepare and evaluate novel intravenous sustained release polymeric nanoparticles for low molecular weight heparin (LMWH). In this study, we prepared intravenously administered polymeric nanoparticles encapsulating LMWH using different grades of PCL (14k, 45k, 80k) and 0.1% PVA solution as surfactant by employing water –in-oil in-water (w/o/w) emulsion and evaporation method. The formulated nanoparticles were evaluated for size, shape, zeta potential, in vitro drug release, and in-vivo biological activity (Anti factor Xa activity) using standard kit, antithrombotic activity in thrombosis induced rat model. Drug and polymer interactions in the nanoparticles were evaluated using XRD.Scanning electron microscopic (SEM) studies on the nanoparticles confirmed the formation of spherical particles with smooth surface. The size of the formed nanoparticles was just about 415-495nm. The % entrapment of nanoparticles was found to be between 71-81%. Nanoparticles showed slow and sustained pattern of release for about 64-78% in 24 hrs. Optimized nanoparticles exhibited excellent improvement in pharmacokinetic parameters and showed good antithrombotic activity when compared to free drug. XRD results demonstrated that the drug changed its physical form in the formulation. The results of this study revealed that intravenous nanoparticles were excellent candidates for sustained drug delivery of LMWH to avoid repeated administration.

Key words: Low molecular weight heparin, Intravenous, Stability, Polycaprolactone, Venous thrombosis, activated partial thromboplastin time.

I. INTRODUCTION:

Low molecular weight heparin is used as an anticoagulant in a variety of clinical situations. It is used as prophylaxis in first line therapy along with aspirin and warfarin and possesses several advantages compared to the other two[1].Use of LMWH can prevent deep vein thrombosis, a blood clot that forms in a vein deep in the body, especially in the lower leg or thigh. Preventing these blood clots can prevent a pulmonary embolism, which is a sudden, potentially fatal, blockage in a lung artery that can occur if the blood clot breaks free and travels through the bloodstream to the lungs. It is potentially a deadly blood clotting condition. According to the National Heart, Lung, and Blood Institute, at least 100,000 cases of pulmonary embolism occur each year in the United States. It is the third most common cause of death among hospitalized patients. This medicine is also used to prevent blood clots in patients confined to bed and also for patients experiencing chest pain and heart attacks. LMWH is used in several other clinical situations including atrial fibrillation, hemofiltration, acute coronary syndrome, indwelling central or peripheral venous catheters. Repeated parenteral (intravenous/ subdermal) injections and therapeutically monitored intravenous infusion are the clinical methods used to deliver LMWH to the patients or in prophylaxis. Despite these several benefits that LMWH offer, the need for repeated parenteral administration is still a major drawback. Further, LMWH has certain clinical and pharmacokinetic limitations[2,3]. It has insignificant oral bioavailability, short half-life, and life-threatening side-effects. To improve the therapy with LMWH, several routes such as oral, transdermal, nasal, pulmonary, parenteral, etc. and several delivery systems such as oral microspheres, oral nanospheres, liposome coated stents, growth factor conjugated heparin nanoparticles, intravenous liposomes, etc. have been investigated.[3, 4]

LMWH also helps in prevention of clots after implantation of a medical device. Generally medical devices tend to form clots upon insertion because of thrombogenecity. For instance, a major

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concern with use of coronary stents is that the stainless steel they are composed of may promote the formation of blood clots, which may occlude the stent and result in heart attack or even death. When a stent is placed in a coronary artery, the body naturally coats the stent with a protective lining called "endothelium", which serves as a barrier that prevents blood clotting. This process generally is complete within three weeks, so patients generally take an anti-blood clotting for four weeks, until the protective lining is in place. LMWH is used for this purpose. Additionally, heparin coated devices are being investigated to weed out this problem of clot formation after implantation. But heparin that is coated onto the devices is faded with time and as a reason with time there is an increased risk of clot formation[5]. A parenteral sustained release dosage form for LMWH can conveniently help the patients in this situation.

Parenteral sustained release dosage forms for LMWH were recently addressed. Jogala et.al, developed intravenous sustained release nanoparticles for LMWH using PLGA (50:50) and tested in an animal model. This study demonstrates excellent results in relation to the particle size, encapsulation efficiency (74.1%) and in vitro sustained release studies demonstrated 75% release in 48 hours. Optimized formulation demonstrated better results with 5- fold enhancement in AUC when compared to free drug solution[6].

Poly caprolactone (PCL) is a type of synthetic, FDA-approved biodegradable polymer, applying in food packaging, tissue engineering, drug delivery, so it becomes one of the most promising biodegradable polymers currently available on the market. PCL have gained enormous prominence because of its commercial availability at reasonable cost, ease of preparation, biocompatibility, versatility, and its hydrolytic degradation products are resorbable, harm-less and practical and market viable drug delivery applications. It is also in the search for appropriate matrices for control drug delivery microspheres and nanoparticles[7,8].

Polycaprolactone based control release systems were recently addressed. Navitha et. al., recently developed 3 mo PCL based drugs-releasing implant for risperidone. In this study, a 12 w steadily sustained release risperidone PCL microsphere without a lag period was investigated using different molecular weight PCLs. Optimized formulation exhibited sustained zero-order release behavior for 90 d without initial burst release and lag phase[7].

Yerragunta et. al., developed 3-month drug releasing risperidone microspheres using blends of PCL. In this study, the developed formulation exhibited 90 d sustained drug release in vitro and in vivo. Furthermore, optimized formulation pharmacodynamics of the drug was measured using locomotor testing; locomotor activity of the optimized formulation was reduced during the study period when compared to the control[8.]

In this study, our goal was to develop novel intravenous sustained release nanoparticles for LMWH and to evaluate various in vitro, in vivo parameters such as pharmacokinetic parameters, activated partial thromboplastin time, thrombotic activity in venous thrombosis rat model. Further, the drug properties in optimized formulations were determined and the results are discussed.

II. MATERIALS AND METHODS

Materials

LMWH (Enoxaparin) was a gift sample from Gland Pharma Pvt. Ltd. (Hyderabad, India). PCL (14k.45k, 80k) were purchased from Sigma-Aldrich Ltd., Germany. Dichloromethane and polyvinyl alcohol were purchased from SD Fine chemicals Ltd. A UV-Visible spectrophotometer from Thermo scientific was used. Probe sonicator (Qsonica. USA), Cooling centrifuge (Hittech, MIKRO 220R, Germany), Freeze dryer, Mini Lyodel (Delvac pumps, Chennai India) were used for the formulation of Nanoparticles. A JSM-5200 Scanning Electron microscope (SEM) Japan, was used to study the surface morphology of Nanoparticles. Zeta sizer Nano ZS (Malvern Instruments, Malvern, UK) was used to measure the particle size and zeta potential of prepared nanoparticles. Fourier transforms infrared spectrophotometer (FTIR) from Perkin-Elmer and X-ray diffractometer (XRD) from PAN Analytical were used. All other ingredients used in this study were of analytical grade.

METHODS:

Preparation of Nanoparticles:

The LMWH nanoparticles were prepared by the water –in-oil in-water (w/o/w) emulsion and evaporation method. The required quantity of drug was taken in 2ml of PVA solution (1%). To this 10 ml of methylene chloride containing the polymer (PCL 14000, PCL 45000, PCL 80000) was added and emulsified with a probe sonicator (Qsonica) for

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30 sec at 60W. The resulting w/o emulsion was then poured in 40 ml of PVA (0.1%) solution and sonicated for 60 sec at 60W using probe sonicator involving in the formation of w/o/w emulsion. This emulsion was kept on magnetic stirrer (15 rpm) for 10 minutes to evaporate methylene chloride. Nanoparticles were then isolated by centrifugation (Hittich, MIKRO 220R), at 18000 rpm for 15 min. The supernatant was removed and assayed to find out entrapment efficiency. The nanoparticles were washed three times with demonized water and subjected to freeze drying (Lyophilizer, MINI –LYODELDELVAC, Chennai, India). The composition of all the formulations mentioned in the Table.1.

Table 1: Composition of Polymeric Intravenous LMWH Nanoparticles

Ingredients	**PNP1**	**PNP2**	**PNP3**
LMWH (mg)	20	20	20
PCL 14000 (mg)	100	---	---
PCL 45000 (mg)	---	100	----
PCL 80000 (mg)	---	---	100
Dichloromethane (ml)	10	10	10
PVA solution (0.1%) (ml)	40	40	40
Water (ml)	2	2	2

Size and surface analysis:

The mean diameter of nanoparticles and their surface potential were evaluated with zeta sizer. Surface morphology was evaluated using scanning electron microscopy (SEM).

Scanning Electron Microscopy: In order to examine the particle surface morphology and shape, scanning electron microscopy (SEM) was used. A concentrated aqueous suspension was spread over a slab and dried under vacuum. The sample was shadowed in a cathodic evaporator with gold layer 20 nm thick. Photographs were taken using a JSM-5200 Scanning Electron Microscope (Tokyo, Japan) operated at 20 kV. The smallest size nanosupension was used for determining surface morphology.

Zeta Potential: The zeta potential is used to measure the electric charge at the surface of the particles, indicating the physical stability of colloidal systems. In this study, the zeta potential was assessed by determining the electrophoretic mobility of the particles. The zeta potential was measured using a Zetasizer Nano ZS (Malvern Instruments, Malvern, UK). Samples were diluted with the respective original dispersion medium which provides information regarding the thickness of the diffuse layer. Diluted nanosuspension was added to the sample cell (quartz cuvette) and was put into the sample holder unit and zeta potential was measured.

Encapsulation efficiency:

The amount of drug entrapped in nanoparticles was determined by turbidimetric assay by measuring the amount of non-entrapped drug in the supernatant recovered after centrifugation. Briefly 1 ml of supernatant was taken to this 1ml of acetate buffer (1M, pH 5) followed by 4 ml of cetylpyridinium solution (0.1%) in Nacl 0.94% and assayed for drug release at 500nm by UV spectrophotometer[6].

In-vitro drug release:

An aliquot of 10 mg of nanoparticles were suspended in flask containing 25 ml of phosphate buffer saline (PBS 0.011M, Nacl 0.15M, pH7.4), and kept it for stirring (150 rpm) on magnetic stirrer. 1mL of sample was taken at

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1,3,6,9,12,18, and 24 hrs using syringe filter. To this 1 ml of acetate buffer (1m pH 5) followed by 4 ml of cetylpyridinium solution (0.1%) in Nacl 0.94% were added and the drug was assayed at 500nm by UV spectrophotometer[6,8].

Selection and Characterization of Optimized Formulation

Using size, zeta potential, encapsulation, in vitro drug release, optimum formulation was selected. The optimized formulation was characterized for various properties.

In-vivo drug release from LMWH nanoparticles:

The in vivo biological activity of LMWH was evaluated by measuring the anti-factor Xa activity with a chromogenic substrate by using standard kit (KRIBIOLISA™ Xa**)** form Krishgen Bio systems according to the method described by the supplier. In vivo biological activity of LMWH was investigated in male wistar rats. All the experiments were conducted according to the guidelines of CPCSEA (Committee for the Purpose of Control and Supervision on Experimental Animals. The animals were divided into 2 groups(n=6). The groups are as below:

Group 1: Intravenous administration of PNP2 formulation

Group 2: Intravenous administration of LMWH solution.

Group 1 received 0.3 ml suspension of PNP2 formulation intravenously. Group 2 received 0.3 ml suspension LMWH solution. All the formulations administered had equal amounts of LMWH. After administration of formulations blood samples were collected at 1,3 6,9, 12,18, 24 hours and the samples were subjected to centrifugation for 10 min at 5000g, using centrifuge (REMI RM 12C), then the plasma collected anti factor Xa activity was measured using standard kit **(**KRIBIOLISA™ Xa**)** form Krishgen Bio systems according to the method described by the supplier[6].

Antithrombotic Activity in Animal Model of Venous Thrombosis

The rats were divided into 3 groups (n=6) with the average weight of the rats was 250g. All the rats were anesthetized by intraperitoneal injection of solution of ketamine (0.15mL), xylazine (0.5 mL) and deionized water (0.75mL) in dose a dose of 0.2mL for 100 g of weight. From the in vitro evaluation PNP2 formulation was selected as optimized formulation. A 4.5 mg/kg of PNP2 formulation was injected intravenously to one group, second group was injected with 4.5 mg/kg LMWH solution and the third group was injected with normal saline solution intravenously. One hour after the administration, the abdomen of the animals was surgically opened and thrombosis was induced according to the modified method. Three hours after thrombosis induction, animals were anesthetized and the abdomen reopened to verify the presence of thrombus[9]. If thrombus was present, it was withdrawn and weighed immediately and after drying for 24h at 37 ^{0}C.

Fourier transform infrared spectroscopy (FTIR):

FTIR spectrum of drug, polymers, physical mixture and formulations were obtained on FTIR instrument. Sample about 2 mg was mixed thoroughly with 100 mg potassium bromide IR powder and compacted under vacuum at a pressure of about 12 Psi for 3 minutes. The resultant disc was mounted in a suitable holder in Shimadzu IR spectrophotometer and the spectrum was scanned over the wave number range of 4000-400 cm^{-1}. IR helps to confirm the identity of the drug and to detect the interaction of the drug with the carriers.

Differential Scanning colorimetry (DSC):

Differential Scanning colorimetry was used to determine drug excipient compatibility studies, and also used to observe more phase changes such as glass transition, crystallization, amorphous forms of drugs and polymers. The physical state of drugs and polymer was analyzed by Differential Scanning calorimeter (Schimadzu). Approximately 5 mg of sample was analyzed in an open aluminum pan, and heated at scanning rate of 10°C/min between 0°C and 400°C. Magnesia was used as the standard reference material.

Powder X-ray diffractometry (PXRD)

The XRD patterns of Drug , polymer , physical mixture and optimized formulation were obtained using X-ray diffractometer (Shimadzu 770). The measuring conditions were as follows: Cu Kα, radiation, nickel filtered; graphite monochromator; 40 kV voltage; and 30 mA current with X'celerator detector. All samples were run at 1^0 (2θ) min^- from 3^0 to 45^0 (2θ).

III. RESULTS AND DISCUSSIONS:

Low molecular weight heparin (LMWH) is the drug of choice for deep venous thrombosis (DVT). This medicine is also considered to be

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better in the treatment of several other disorders. Despite these several benefits that LMWH offer, the need for repeated parenteral administration is still a major drawback. Further, LMWH has certain clinical and pharmacokinetic limitations. It has insignificant oral bioavailability, short half-life, and life-threatening side-effects. To improve the therapy with LMWH, several routes such as oral, transdermal, nasal, pulmonary, parenteral, etc. and several delivery systems such as oral microspheres, oral nanospheres, liposome coated stents, growth factor conjugated heparin nanoparticles, intravenous liposomes, etc. have been investigated[10.11].

This study particularly focused on the formulation of intravenously administered sustained release nanoparticles of LMWH to treat various diseases like venous thromboembolism which includes venous thrombosis and pulmonary embolism.

Intravenous nanoparticles for heparin, a molecule structurally like LMWH have been recently reported by Shahbazi et al.[10]. In this study they developed chitosan-heparin intravenous polymeric nanoparticles intended for the prolonged delivery of heparin. The nanoparticle preparation method was optimized using taguchi orthogonal array. The study performed ex-vivo and in-vivo tests in humans and rats respectively using activated partial thromboplastin time biological index. The study has shown good results in relation to in-vitro drug release and excellent aPTT values in both the cases of ex-vivo as well as in-vivo. However these studies were performed only for heparin and did not evaluate the anti-factor Xa activity and antithrombotic activity. Thus, intravenous administration of LMWH is a novel approach for extended and enhanced activity.

In this study, Polymeric intravenous nanoparticular formulation for sustained release of LMWH was prepared, characterized, and tested in animal model. Polymeric intravenous nanoparticular formulations were prepared by the water-in-oil in-water (w/o/w) emulsion and evaporation method. The prepared nanoparticles were evaluated for particle size, zeta potential, surface morphology, in vitro drug release and in vivo anti factor xa activity, Anti thrombotic activity in venous thrombosis induced rat model. Determining the levels of ant factor Xa activity in plasma is an indication of drug release from the formulations. This is a routine assay used for determination of heparins in the plasma after various administration modes.

Drug and Excipient compatibility studies were conducted using FTIR, DSC, XRD. To formulate polymeric nanoparticles we used poly caprolactones (14000, 45000, 80000).The FTIR spectra of drug and PCL 45k and polymer were compared with the FTIR spectra of optimized formulation. FTIR results suggested that polymeric particles were formed and there was no chemical interaction between drug and polymers used. The presence of peaks at the expected range confirmed that the materials taken for the study are genuine and there were no possible interactions occurred.

All the fabricated nanoparticle formulations were evaluated for particle size, zeta potential, encapsulation efficiency and the results were represented in Table.2. The particle size of all the formulations was found between 415- 490nm.

Nanoparticle formulations exhibited negative surface charges of - 17.4, - 23.5, -26.1 respectively, due to the presence of PVA and Polycaprolactones and LMWH, all the fabricated nanoparticles were exhibited negative surface charges, here PVA works as stabilizer and increases the stability of all the nanoparticle formulations[6].The study of SEM was conducted to confirm the formation and surface morphology of nanoparticles. All the nanoparticles were spherical in shape with a smooth surface. (Figure.1).

The Encapsulation efficiency of the prepared particles was determined by turbidimetric assay method. Encapsulation efficiencies of the nanoparticles PNP1, PNP2, PNP3 are 69±0.45, 84±0.19, 76±0.25 respectively. The maximum encapsulation efficiency (84±0.25) was exhibited by PNP2 nanoparticles.

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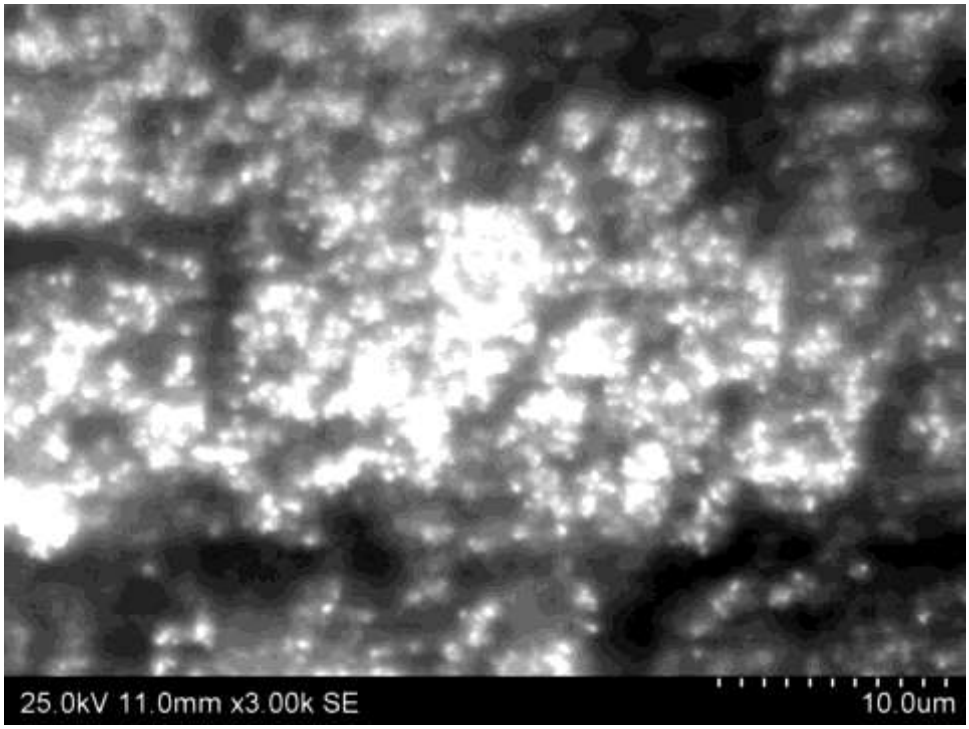

Figure 1. Scanning Electron Microscope pictures of the Nanoparticles

Table 2. Particle size, zeta potential and Encapsulation efficiencies of all nanoparticle formulations

Formulation	Particle Size (nm)	Zeta Potential (mv)	Encapsulation efficiency (%)
PNP1	425±1.21	-17.4±0.8	69±0.45
PNP2	415±2.12	-23.5±0.6	84±0.19
PNP3	490±3.35	-26.1±0.7	76±0.25

In vitro drug release of the, all the three formulations were performed in 25 ml of phosphate buffer saline (PH7.4) as per the method described above. The release profiles are shown in Figure. 2. All the formulations sustained the release of the drug for a period of 24 h. PNP2 formulation exhibited slow and sustained release pattern and maximum drug release is about 78% in 24h. This must be due to the hydrophilic nature and its high crystallinity of the polymer (PCL). High crystallinity in the particles can lead to slow rate of drug release in the sustained pattern. From the results of in vitro evaluation of the nanoparticles, PNP2 formulation was selected and further subjected to in vivo characterization.

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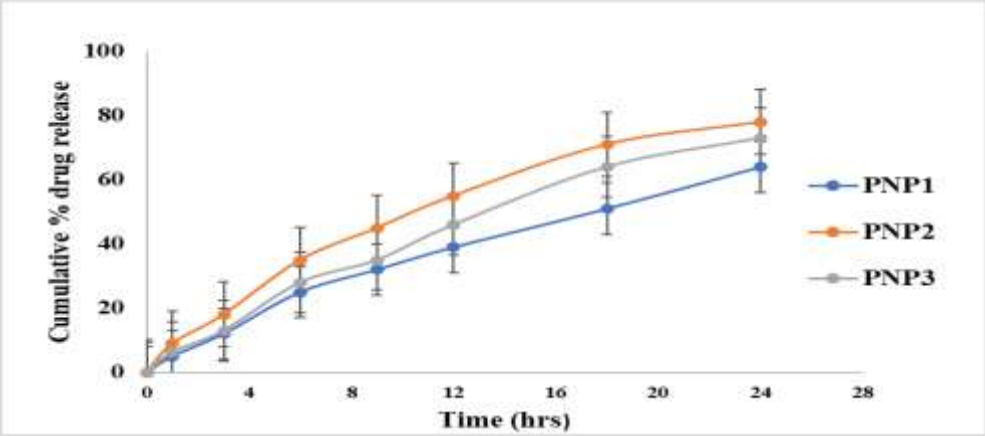

Figure 2. In-vitro drug release profiles of all the formulations (PNP)

Based on the data obtained from in vitro characterization, an optimized formulation (PNP2) was selected, and this was further subjected to in vivo evaluation in rats to estimate in vivo drug release by measuring anti factor X_a activity. Plasma concentration vs time profile of all the fabricated formulations and pure drug solution are represented in Figure 3. Software was used to calculate the pharmacokinetic parameters, and the results were represented in Table 3. From all the formulations, PNP2 formulation exhibited good results in relation to all pharmacokinetic parameters. For pure drug peak plasma concentration (C_{max}) was 14.60 µg/ml, T_{max} was 1h, AUC0-∞ was 39.46 (µg ml-1 h-1) and after 6 hours concentration was 0.3µg/ml. F2 formulation shown prolonged plasma concentration for 24 hrs, peak plasma concentration was 15.4 µg/ml and T_{max} was 15h. After 24 hrs concentration of drug in plasma was 2.4 µg/ml. In comparison to pure drug solution PNP2 formulation exhibited the four- fold enhancement (39.46 vs 158.20 µg ml-1 h-1) in area under curve (AUC0-∞). The results demonstrated that low molecular weight heparin sustained release from encapsulated nanoparticles (F2) for 23 hrs, by maintaining minimum concentration of drug (2.4µg/ml) required for preventing venous thrombosis. Considerable difference was observed in the plasma concentrations at each time point.

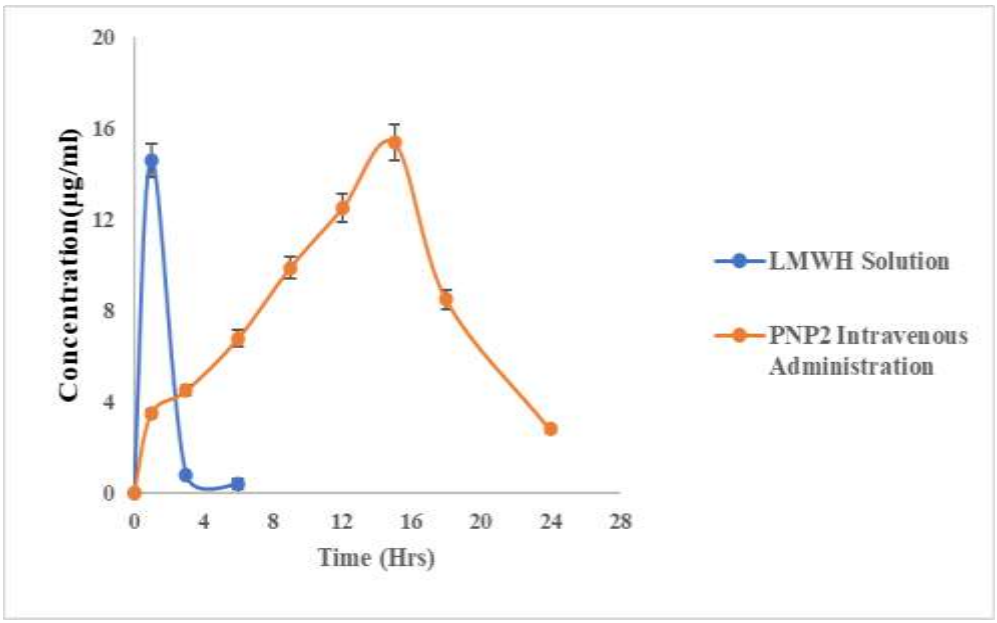

Figure 3. Plasma concentration profiles of optimized formulation (PNP2) and LMWH solution.

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Table 3: Pharmacokinetic parameters of LMWH solution and PNP2 optimized nanoparticle formulation

Pharmacokinetic Parameter	LMWH Solution	PNP2
C_{max}(µg/ml)	14.6	15.4
T_{max}(hrs)	1	15
AUC0-α(µg ml^{-1} h^{-1})	39.46	158.2

Antithrombotic activity of the optimized formulation (PNP2) and the pure drug solution, control (normal saline solution) determined in venous thrombosis induced rat model. The weight of the thrombi formed after intravenous administration of control, pure drug solution, and optimized formulation was to be 22.10±0.13, 4.2±0.21,0.9±0.1 mg respectively. The weight of the thrombi formed in the group of rats which received the optimized formulation was very less when compared to the weight of the thrombi formed in the groups of rats which received the pure drug solution and normal saline solution. This is probably due to the maintenance of the minimum of the concentration of the drug (0.26 µg/ml) in the plasma for a prolonged period which requires for the venous prevention thrombosis. The results were represented in Figure 4.

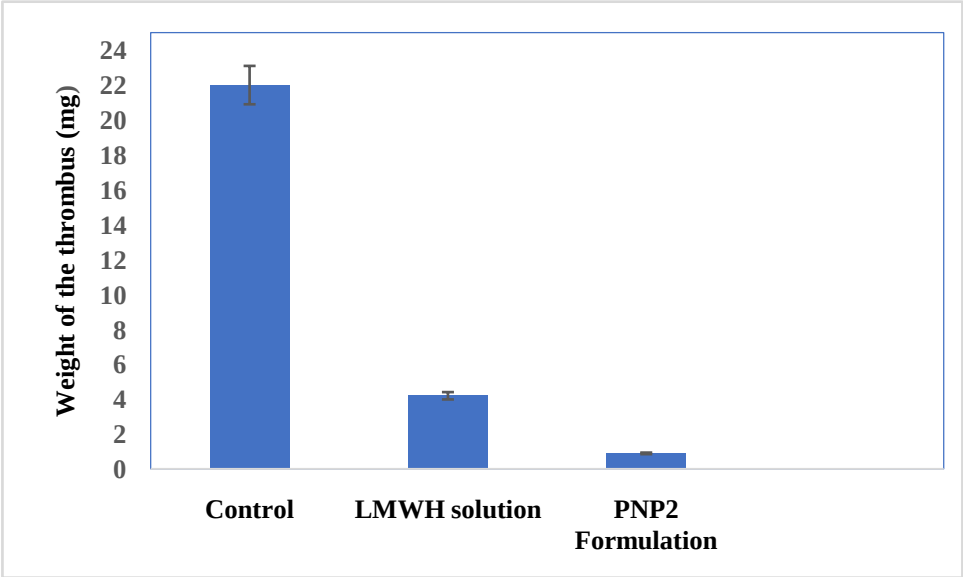

Figure 4. Weight of thrombus after intravenous administration of control, free drug, and Optimized formulation (PNP2). (Indicates mean and standard deviation of the weight)

FTIR spectra of the drug, polymers and optimized formulation were recorded in the range of 4000-400 cm- . The FTIR spectra of drug and PCL 45k compared with the FTIR spectra of the optimized formulation. The characteristic functional groups of drug and PCL 45k showed peaks in the following wave number. In pure drug there was C-O stretching at 1230 cm- , N-H stretching at 3350 cm- , N-H bending at 1624 cm- . S=O peak at 1145 cm- . In PCL 45K characteristic broad peak of O-H was there at 3500-3600 cm- . C-H stretching sharp peak at 2850 cm- . There was the appearance of same characteristic peaks are appeared in the optimized formulation this indicates there was no chemical interaction between drug and polymers used (data not shown). The optimized optimized formulation(PNP2) was subjected to DSC and PXRD studies. DSC studies indicated that there was a change in the solid state of the drug upon formulating into nanoformulation. XRPD results yielded interesting results. The drug was crystalline due to the sharp peaks demonstrated in XRPD. With the optimized nanoparticles, there was a bump in the peak suggesting conversion of LMWH to less crystalline form and that may be due to amorphous form (Figure.4)

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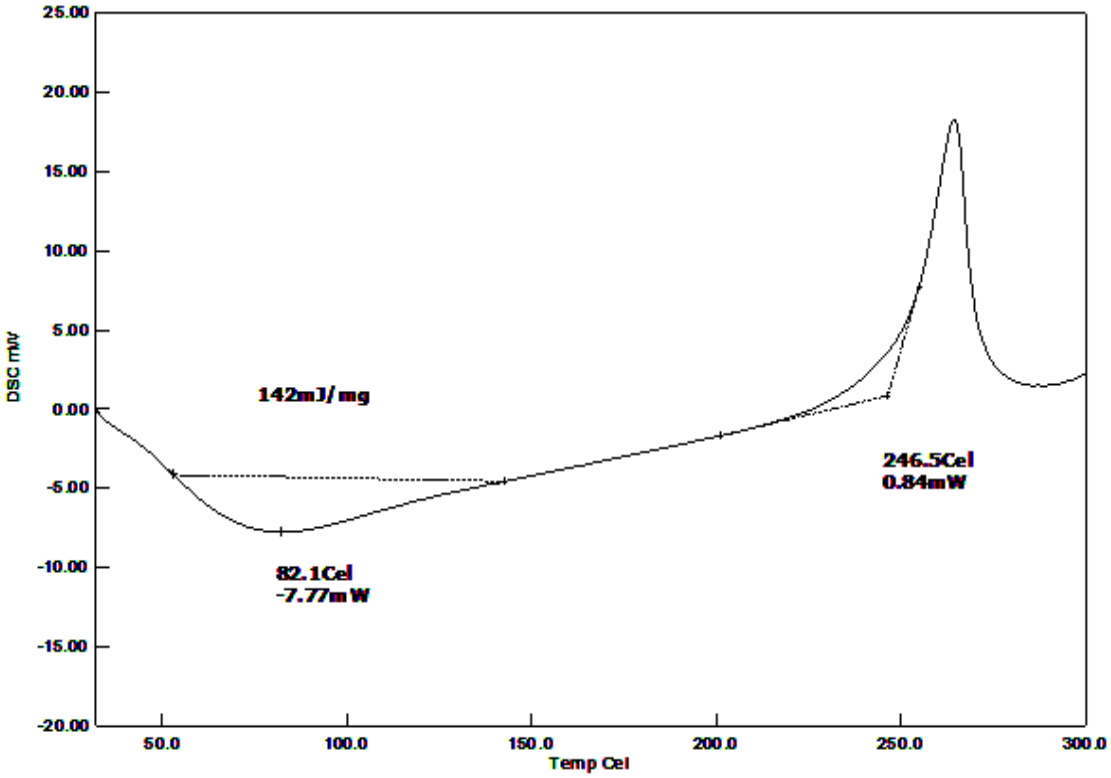

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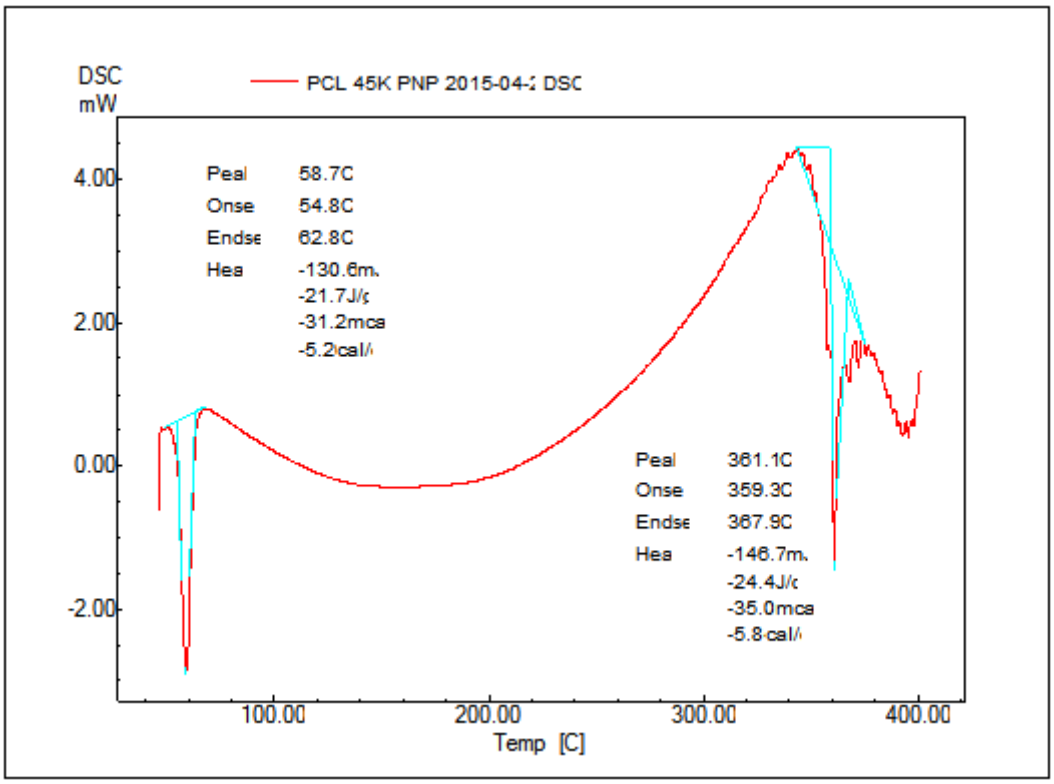

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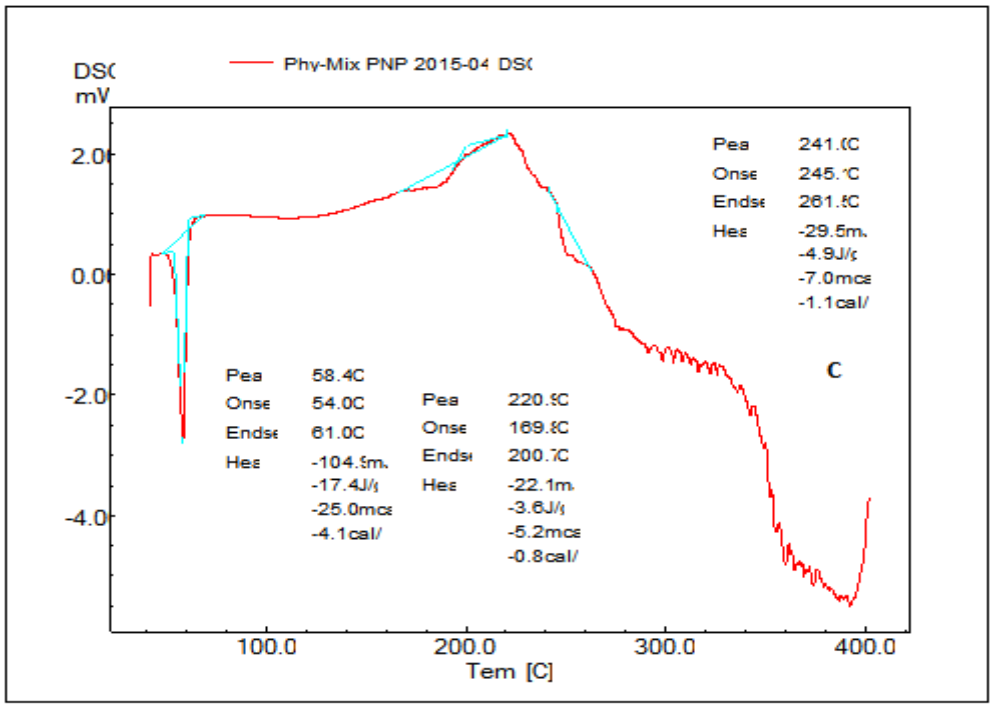

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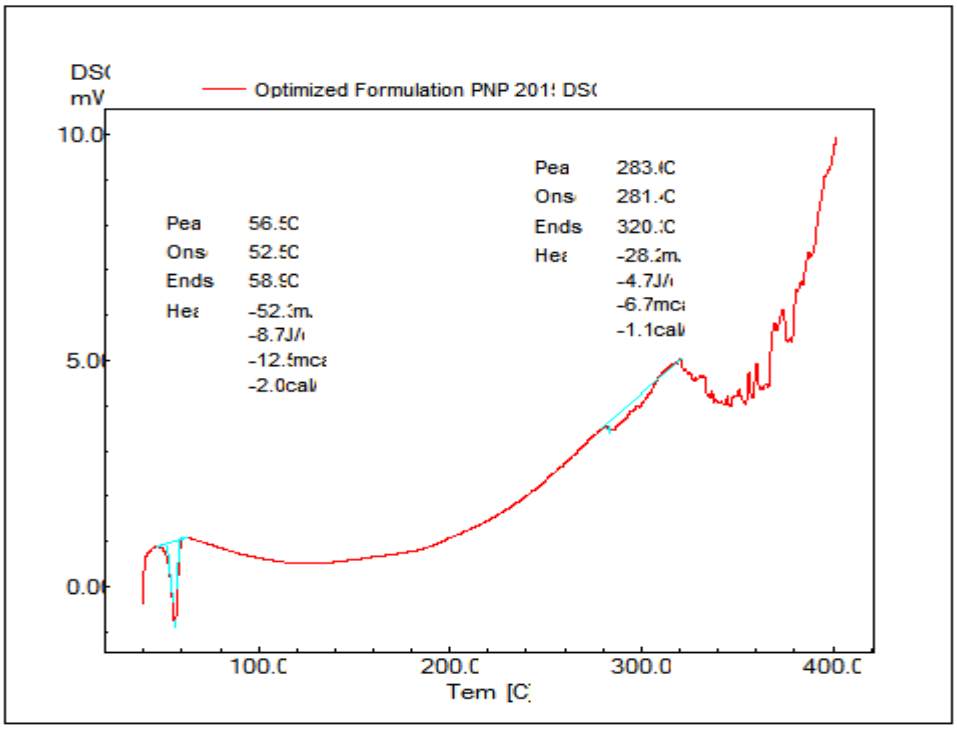

(D)

Figure5. DSC thermograms of various samples to demonstrate the solid state of the drug in the formulation (A: Pure LMWH; B: PCL45K; C: Physical Mixture; D: Optimized Formulation).

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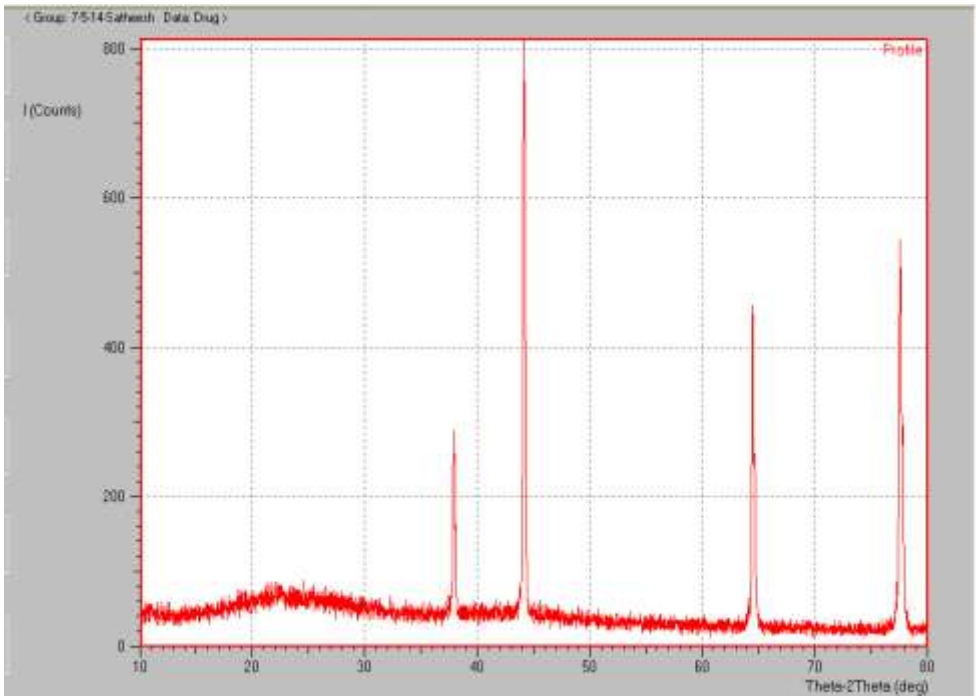

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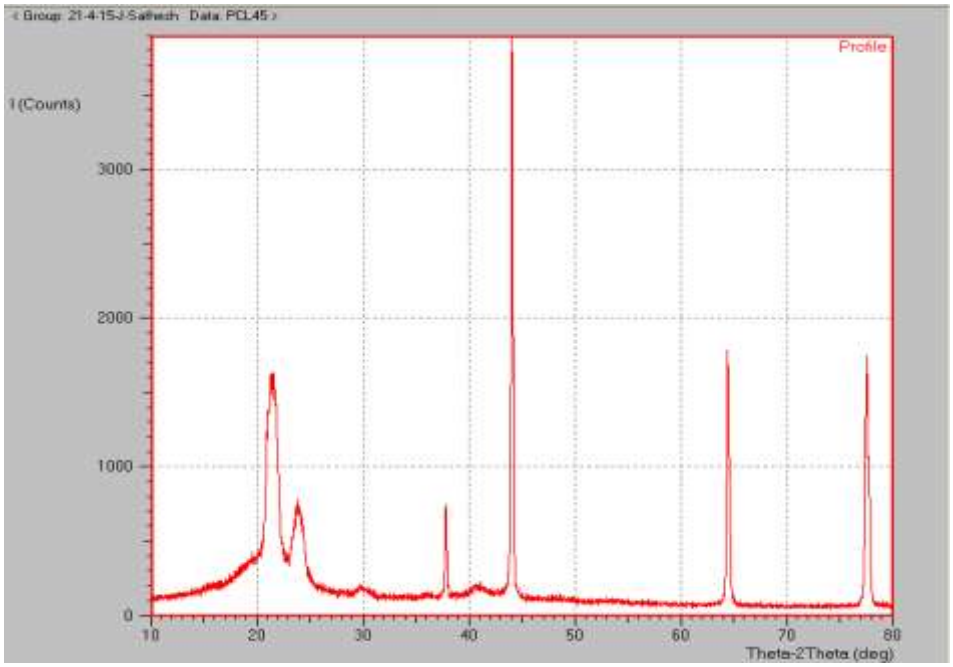

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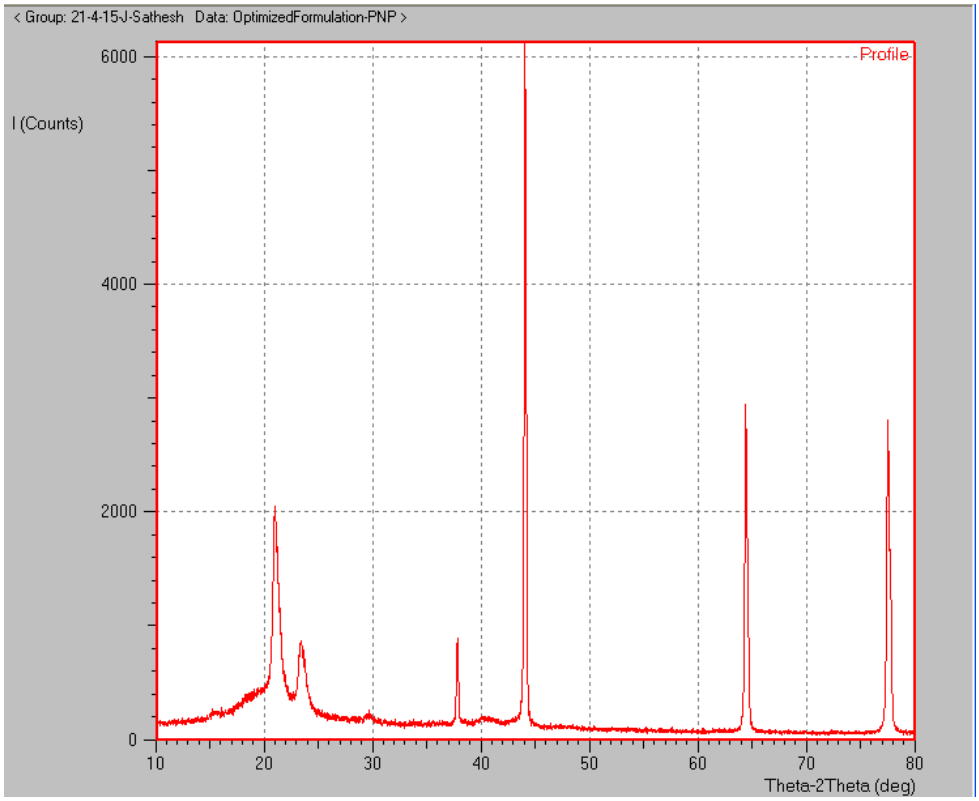

(C)

Figure 6. XRPD Patterns of LMWH (A: Pure Drug; B: PCL 45000 ; C:Optimized Formulation)

IV. CONCLUSION:

In this study, we have fabricated novel and better intravenous sustained release nanoparticles for low molecular weight heparin (LMWH), optimized the formulation so as to demonstrate excellent results in relation to the particle size, encapsulation efficiency (84%) and in vitro sustained drug release -for 24 h with about 78 % drug release. Optimized polymeric intravenous nanoparticle exhibit 4-folds enhancement in AUC when compared to a free drug solution. Furthermore, optimized formulation demonstrated excellent antithrombotic activity in venous thrombosis rat model. Based on the above results, these fabricated nanoparticles would serve as a promising prospect for the efficient prevention of venous thrombosis. The results were tempting for the clinical realization of the formulation and can be taken forward for further development.

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